SOME ASPECTS OF RESPIRATORY METABOLISM DURING METAMORPHOSIS OF NORMAL AND DDT-RESISTANT HOUSE FLIES, MUSCA DOMESTICA L.

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In this study, the respiratory metabolism of two strains of house fly, one of them resistant to DDT, has been investigated in order to ascertain some of the biochemical events associated with developmental processes, and to contribute to our scant knowledge of the mechanism of insect resistance to insecticides.

Inasmuch as both DDT and cyanide are known to inhibit cytochrome oxidase, it seemed worthwhile to compare the effects of cyanide on normal and resistant strains of a single species. Pupae were used in these experiments because measurements of their oxygen consumption could be made without the interference of bodily activity, and also because they provide an opportunity for gaining a better understanding of biochemical events during metamorphosis.

General reviews of the respiratory metabolism of insects during this stage of development have been compiled by Needham (1942) and Wigglesworth (1947). The inhibitory action of cyanide, azide and carbon monoxide on cytochrome oxidase has been studied by Keilin and Hartree (1939) and by others. With insect material, the effects of one or more of these inhibitors have been reported for eggs of the grasshopper, *Melanoplus differentialis* (Bodine and Boell, 1934); for larvae of the codling moth, *Cydia pomenella* (Graham, 1946); and for pupae of *Drosophila melanogaster* (Wolsky, 1938). Collier (1940) and Zukel (1944) studied the inhibition of cytochrome oxidase by phenothiazine, while Sacktor (1949) and Johnston (1950) found that DDT also inhibits this enzyme.

The development of insect resistance to insecticides has become a problem of major economic and, potentially, medical importance. Quayle (1916) and others showed that certain scales had become resistant to cyanide. Cyanide resistance has been reported also for *Drosophila melanogaster* and an aphid, *Aphis gossypii* (Boyce, 1928), as well as for the confused flour beetle, *Tribolium confusum* (Gough, 1939). Screw-worms, *Callitroga americana*, resistant to phenothiazine were found by Knipling (1942). In recent years, reports of house flies and other insects resistant to DDT (1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane) and other synthetic insecticides have become commonplace. The subject has been reviewed by Babers (1949). Since then, experiments by Sternburg and Kearns (1950) and Sternburg, Kearns and Bruce (1950) revealed that all stages of several DDT-resistant strains of house fly were capable of converting DDT to non-toxic DDE (1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene), whereas only pupae of a normal strain could metabolize DDT to DDE. In partial agreement with the above results, Perry and Hoskins (1950) found that adults of both a resistant and normal strain could detoxify DDT but that the extent of this detoxification was greater with the resistant strain. Despite these observations, it may still be said that

little is known about the physiological mechanisms concerned in resistance to insecticides, since the mode of action of the compounds themselves is seldom understood.

Methods

The DDT-resistant strain of house flies was derived from the Ellenville line. Details concerning this stock and the rearing procedure have been reported elsewhere (Sacktor, 1951).

Warburg manometers were used for determinations of normal and cyanide inhibited oxygen consumption. Four pupae of a given age were employed per determination. In measuring the normal oxygen consumption the pupae were placed in the main compartment of the flask, the center well containing 0.2 ml. of 0.5 *M* KOH. To determine the consumption during cyanide inhibition, the pupae were placed in the side arm of the vessel. As recommended by Robbie (1946), the center well contained KCN-KOH or $Ca(CN)_2$-$Ca(OH)_2$ mixtures, depending on the experimental concentration of cyanide used. The former mixture was used for determinations of the inhibition with 3×10^{-4} *M* cyanide, the latter with 4×10^{-3} and 5×10^{-2} *M*. When measuring the inhibition with the lowest cyanide concentration, the main compartment of the flask contained 1.0 ml. of *M*/150 phosphate buffer, pH 7.0, and 3×10^{-4} *M* KCN. Nothing was placed in the main compartment when determining the inhibition with higher cyanide concentrations.

The $Ca(CN)_2$-$Ca(OH)_2$ mixtures were prepared according to the procedure of Robbie and Leinfelder (1945). The HCN concentration of these mixtures was determined by the method of Liebig (1851). The experimental HCN concentrations were interpolated or extrapolated from the data given by Robbie (1946).

The flasks were allowed to equilibrate for 30 minutes at 30° C. with a shaking rate of 120 per minute. The oxygen uptake was thereafter recorded every 10 minutes for one hour.

Cytochrome oxidase activity was measured in a Beckman spectrophotometer at 5500 Å. The procedure described in detail in a previous paper (Sacktor, 1951) was followed with but one minor modification: For each determination 5 pupae were homogenized in 5.0 ml. iced distilled water. A 1.0 ml. aliquot of this stock homogenate was diluted five-fold, and paired determinations were made using 0.5 ml. of the diluted homogenate.

To determine the mortality of pupae exposed to cyanide vapor, the pupae were collected from the Warburg flasks immediately after respiration studies. These pupae were then washed, dried and placed in petri dishes. The non-emergence of the flies was used as the death criterion.

Results

The effect of cyanide at various concentrations on the oxygen consumption of normal pupae is shown in Figure 1. Each point on the curves representing the normal oxygen consumption and that of pupae exposed to 0.3×10^{-4} *M* cyanide indicates the average of 12 determinations. The remaining points represent averages of from 4 to 12 determinations each. The per cent of respiration insensitive to cyanide varied with the age of the pupae, as recorded in Table I.

TABLE I

Per cent of pupal respiration insensitive to cyanide

Age days	Normal strain			Resistant strain		
	M conc. of HCN					
	3×10^{-4}	4×10^{-3}	5×10^{-2}	3×10^{-4}	4×10^{-3}	5×10^{-2}
0	16	8	6	19	14	8
1	38	36	21	72	69	61
2	45	39	32	67	46	68
3	73	31	10	87	61	37
4	83	24	15	65	25	18

Similar data for the DDT-resistant strain are given in Figure 2 and Table I.

Figure 3 shows the change in cytochrome oxidase activity in both strains of pupae in relation to their development. Each point represents the mean and its standard error for 14 to 26 determinations.

In Table II is shown for both strains of pupae the mortality due to exposure to different cyanide concentrations. These results were obtained by combining those pupae, 16 to 48 in number, used under each experimental condition.

TABLE II

Per cent mortality of pupae exposed to cyanide vapor

Age days	Normal strain			Resistant strain		
	M conc. of HCN					
	3×10^{-4}	4×10^{-3}	5×10^{-2}	3×10^{-4}	4×10^{-3}	5×10^{-2}
0	94	100	100	95	100	100
1	25	92	100	9	69	100
2	36	100	100	22	100	100
3	75	100	100	22	88	100
4	88	100	100	29	96	100

DISCUSSION

Oxygen consumption

The oxygen consumption of normal and resistant house fly pupae exhibited the characteristic U-shaped curve during their development. These results are in agreement with a multitude of other investigations with holometabolic insects (Haub and Hitchcock, 1941, with Phormia; Frew, 1929, with Calliphora; Wolsky, 1938, with *Drosophila melanogaster;* Dobzhansky and Poulson, 1935, with *D. pseudoobscura;* Ludwig, 1931, with Popillia; Lindgrem, 1935, with Tribolium; and others).

Figures 1 and 2 reveal that the oxygen consumption of the white pre-pupae (zero age) was considerably higher then than it was immediately prior to the

emergence of the adults. This is an apparent disagreement with some other investigations. Oxygen consumption of the early stages, however, was measured in these experiments within one-half hour after puparium formation. This is as much as 10 hours earlier than has been reported by some other investigators. It is

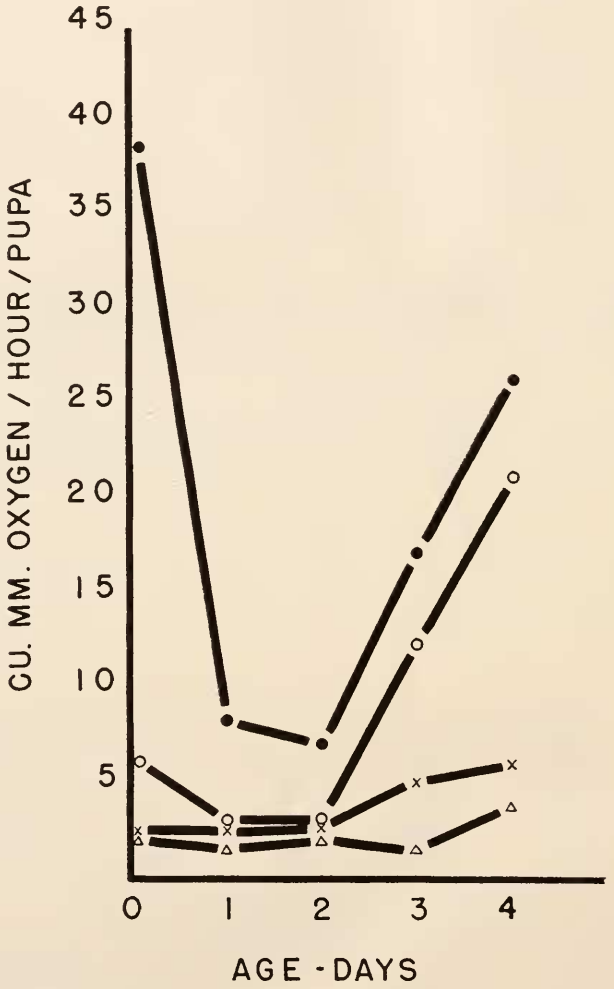

FIGURE 1. The effect of cyanide at various concentrations on the oxygen consumption of normal house fly pupae. Solid circles represent normal oxygen consumption. Open circles, crosses and triangles represent oxygen consumption in presence of, respectively: 3×10^{-4} *M*, 4×10^{-3} *M*, and 5×10^{-2} *M*, cyanide.

thus evident that oxygen consumption must decrease rapidly at this critical period. This interpretation is in agreement with measurements of oxygen consumption in *Drosophila pseudoobscura* by Dobzhansky and Poulson (1935), and with the unpublished data of Bodenstein and Sacktor on larval and pupal cytochrome oxidase in *D. virilis,* and of Levenbook (1950) on respiration in Calliphora.

The significance of the U-shaped curve is not fully understood. Gaarder (1918) has shown that the fall in oxygen uptake is not due to a reduction in the oxygen tension in the tissues. As pointed out by Needham (1942), the idea "that it (the change in oxygen uptake) represents the metabolic consequences of

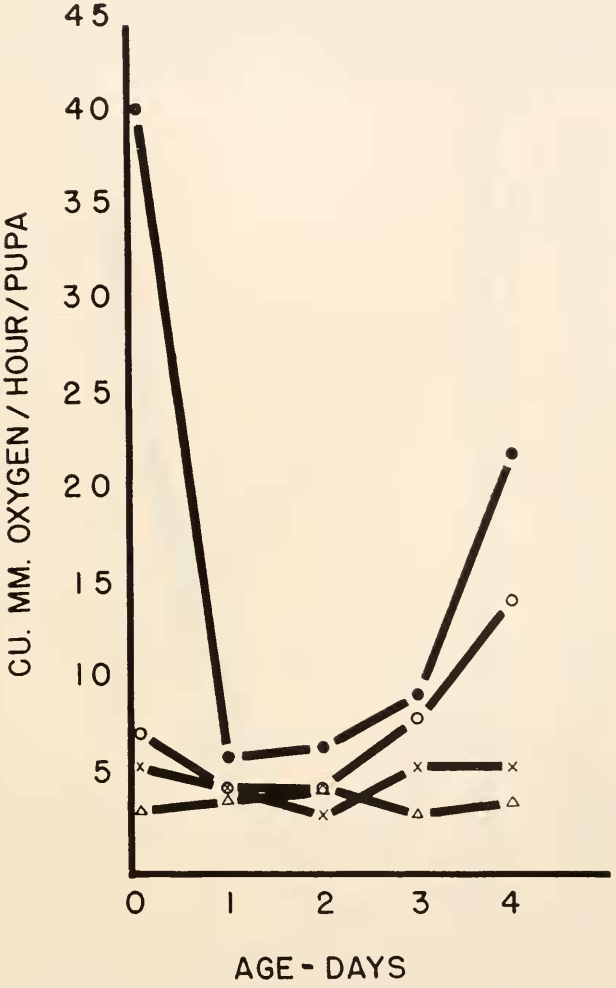

FIGURE 2. The effect of cyanide at various concentrations on the oxygen consumption of DDT-resistant house fly pupae. Solid circles represent normal oxygen consumption. Open circles, crosses and triangles represent oxygen consumption in presence of, respectively: 3×10^{-4} *M*, 4×10^{-3} *M*, and 5×10^{-2} *M* cyanide.

complete histolysis followed by new tissue differentiation . . . cannot be true." Such a view, which was once widely accepted, is not consistent with the histological data found by Dobzhansky and Poulson (1935) for Drosophila pupae. Agrell (1947) assumed that the U-shaped course of respiration is, to a certain degree, connected with variations in the activity of the dehydrogenase systems. Wolsky

(1938) attributed the changes in oxygen consumption to the quantity or activity of the cytochrome system. The results of the present study support Wolsky's interpretation in part, but point also to additional complicating factors.

Cytochrome oxidase

The cytochrome oxidase activity of both strains changes during pupal development. The activity follows a U-shaped curve (Fig. 3), which has, in general, a

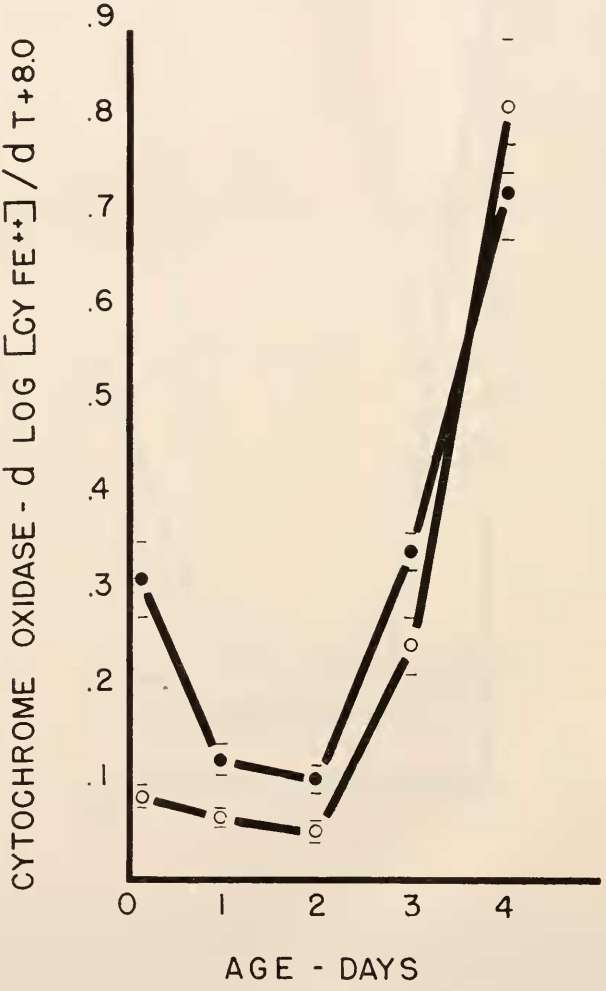

FIGURE 3. The change in cytochrome oxidase activity in both strains of house fly in relation to their pupal development. Solid circles represent normal strain; open circles represent DDT-resistant strain.

similar appearance to that of oxygen consumption *in vivo*. The relationship of respiration to cytochrome oxidase activity is shown in Figure 4 (Boell, 1945, found a like comparison with developing embryos of *Amblystoma punctatum*).

The broken line drawn through the origin is based on the assumption of 1 : 1 correspondence between oxygen uptake and cytochrome oxidase activity. In plotting these data, the total measured oxygen consumption was corrected by subtracting the average cyanide insensitive respiration of 2.0 cu. mm./hr./pupa for the normal strain and 4.0 cu. mm./hr./pupa for the resistant strain. The graph shows that an approximately linear relationship exists between cyanide sensitive respiration and cytochrome oxidase activity during pupal development for 1–4 days of age.

It will be noted from Figure 4 that some of the points reveal that there is slightly more cyanide sensitive respiration than is accountable by cytochrome

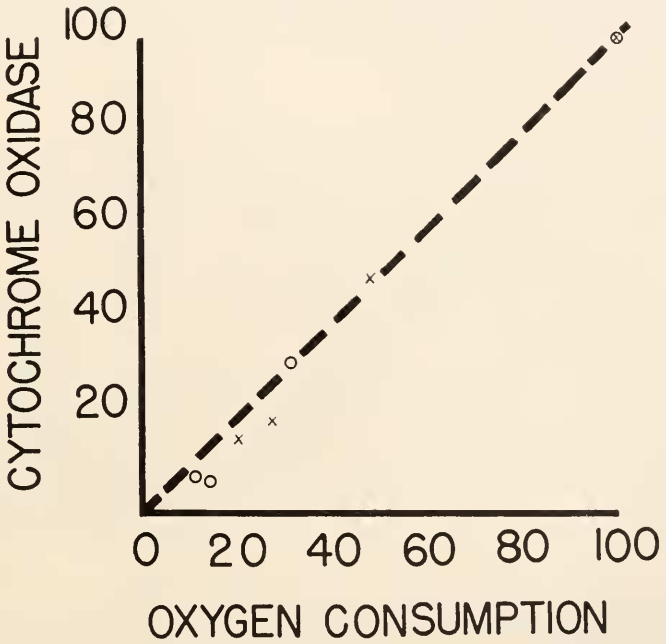

FIGURE 4. The relationship of oxygen consumption to cytochrome oxidase activity. All numbers in per cent of the highest O_2 consumption and oxidase activity observed. Crosses represent normal strain; open circles represent DDT-resistant strain. For further details see text.

oxidase activity. An examination of Figures 1 and 2 shows that these points correspond to pupae one and two days old. Further, the data obtained for zero age pupae are not included in the curve of Figure 4. These data (compare Figs. 1 and 3) indicate that there is at this stage a great excess of cyanide sensitive respiration not accounted for by cytochrome oxidase, and thus suggest that other cyanide sensitive systems are contributing to the total oxygen consumption. Such systems might involve tyrosinase or catalase, which are cyanide sensitive enzymes likely to be present in animal tissues. But from the existing evidence (Fink, 1930; Williams, 1936) there seems to be little correlation between catalase content of insects and the intensity of their respiration. Dennell (1947), however, related tyrosinase activity to puparium formation. In his review of tyrosinase in insects,

Sussman (1949) reported that tyrosinase activity increased until pupation, whereupon there was a rapid decrease.

Thus, the present experiments provide some evidence partially substantiating Wolsky's hypothesis, that changes in oxygen consumption during metamorphosis are related to the activity of cytochrome oxidase. But there are other factors here, in addition to the cytochrome system, which contribute to the total respiration. These are a cyanide insensitive system (which will be discussed later) and another cyanide sensitive system, perhaps tyrosinase. The latter is significant mainly in the early stages of pupal development.

The factors that cause changes in the cytochrome oxidase activity are not known, and additional experiments, some of which are underway, are necessary in order to ascertain the significance of these changes.

Inhibition by cyanide

Cyanide inhibition experiments (Commoner, 1940; Robbie, 1949; and others) reveal considerable variation among organisms in regard to their cyanide sensitive and insensitive systems. Runnström (1930) with the sea urchin and Bodine and Boell (1934) with the grasshopper showed that cyanide had various effects on different embryonic stages. It is apparent from Figures 1 and 2 and Table I that in house fly pupae the magnitude of inhibition by cyanide is dependent on the the developmental stage.

Upon complete inhibition of the cyanide sensitive respiration, the cyanide stable respiration (in cu. mm./hr./pupa) was approximately constant throughout development. As seen from Figure 5, the per cent inhibition was therefore found to be dependent upon the original cyanide-free rate of respiration. Only one deviation was observed, *i.e.*, with 4 day old pupae of the normal strain. As can be seen from Figure 1, this deviation can best be explained by the apparent failure of 5×10^{-2} *M* cyanide to inhibit completely the cyanide sensitive respiration of that stage.

It is also evident that the effects of submaximal cyanide concentrations depended upon the developmental stage of the pupae, for inhibition at a given concentration of cyanide decreased markedly in the later stages. Similar results were obtained by Wolsky (1938), with carbon monoxide and Drosophila pupae. In general terms, he proposed two explanations, namely; "(1) there may be changes in the physical-chemical properties of the medium in which the Warburg-Keilin system is reacting, affecting the velocity constants of the reactions or the solubility of the gases; (2) there may be a qualitative change in the Warburg-Keilin system, which alters the velocity constants of its reactions, so that it reacts more readily with O_2 and less readily with CO." He also noted that the work of Szorenyi and Tschepinoga (1936), who reported that in trained muscles the oxygen consumption increased and at the same time the respiration became more resistant to cyanide.

This partial inhibition by cyanide and carbon monoxide is of considerable interest and may reveal certain properties of the Warburg-Keilin system. Unfortunately the possible explanations are based partly upon conjecture, but a discussion of them may be worthwhile in directing future experimentation. Cook *et al.* (1931) and Cook and Haldane (1931) showed that with bacteria, at a given level

of cyanide or carbon monoxide, the sensitivity of the respiration was dependent on the nature of the substrate. They observed that the affinity constant (K = affinity of oxidase for O_2/affinity of oxidase for CO) with glucose was approximately 8 ×, 4 × and 2 × that when formate, succinate or lactate, respectively, were used. A similar effect was found by Ogston and Green (1935) for the respiration of yeast in various substrates. At a given cyanide level, the greatest oxygen consumption was with glucose as the substrate. The consumption decreased, in this order, when glycerophosphate, lactate, hexosediphosphate and hexosemonophosphate were used. There is, therefore, evidence that the cyanide

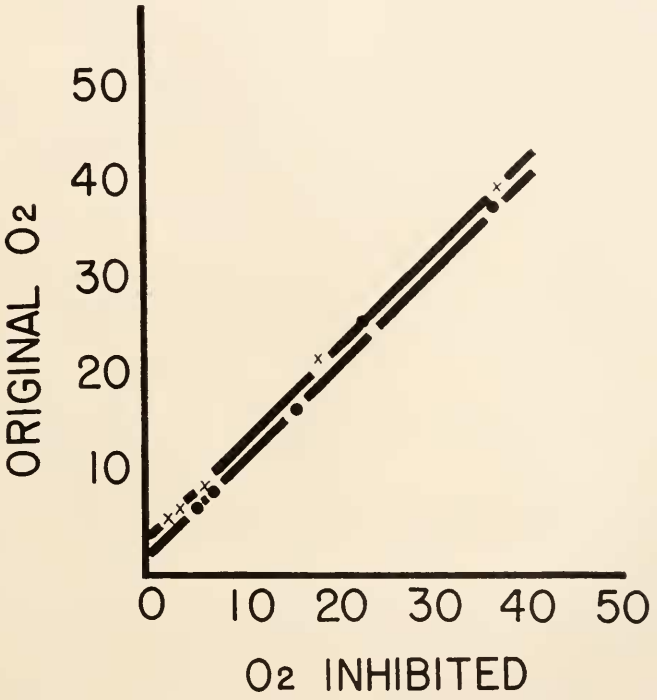

FIGURE 5. Amount of oxygen inhibited as a function of the original oxygen consumption. Ordinate, normal O_2 consumption in cu. mm./hr./pupa. Abscissa, amount of O_2 in cu. mm./hr./pupa inhibited by cyanide. Crosses represent DDT-resistant strain; closed circles represent normal strain. For further explanation see text.

(or carbon monoxide) sensitivity of the oxidation of various substrates is a function of the relative capacity of their specific dehydrogenases. Some dehydrogenases appear to require a greater degree of activity on the part of the oxidase, thus rendering the respiratory rate more sensitive to the decrease in oxidase activity by inhibitors.

During metamorphosis the developing insect utilizes reserve oxidizable substances. In many cases glycogen appears to be utilized throughout metamorphosis (for more details see Rockstein, 1950). As shown by Ludwig and Rothstein (1949) in the Japanese beetle, *Popillia japonica*, glycogen decreases most rapidly

during the first days of pupal life. Glucose also may serve as a substrate during the pupal stage. Evans (1932) found that the glucose content of the blowfly, *Lucilia sericata,* falls in young pupae but remains fairly constant during the later stages. A continuous utilization of glucose during metamorphosis occurred in the mealworm, *Tenebrio molitor,* (Evans, 1934). Courtois-Drilhon (1931), Crescitelli and Taylor (1935) and Ludwig and Rothstein (1949), however, have shown an increase in glucose during the prepupal and pupal period in several species of Lepidoptera and the Japanese beetle. This increase in glucose content is attributed to its conversion from glycogen. There are considerable differences in the utilization of fat during metamorphosis of insects. In general, fats seem to be used primarily in the late stages of development (Frew, 1929; Evans, 1934; and Ludwig and Rothstein, 1949).

The above data reveal that the substrates metabolized during metamorphosis vary according to the developmental stage and with the species studied. In general, carbohydrates are used primarily during the early stages whereas fats are consumed later. Since there is evidence that sensitivity to cyanide is dependent upon the nature of the substrate, the differences found in the effects of submaximal cyanide concentrations on developing house fly pupae may perhaps be related to changes in the substrate being metabolized. Although this inference is supported by analogy with facts determined in studies of other organisms, investigations suitable for testing this hypothesis are yet to be made with the house fly.

Another explanation, although related to the above, of the differences in effects of submaximal cyanide concentrations is the possibility that partial cyanide inhibition may cause a change in the hydrogen donators. Such alterations may even result in a stimulation of oxygen consumption. This possibility is excellently discussed by McElroy (1947) and for details it is recommended that reference be made to his paper. He has shown that the oxygen consuming reactions exhibited by normal tissues may be entirely different from those which maintain the residual respiration during partial inhibition.

Cyanide, in addition to inhibiting cytochrome oxidase, may also react with other metabolic participants and thus have an effect on the oxygen consumption *in vivo.* As shown by Marshall and Rosenfeld (1934), cyanide combines with aldehydic and ketonic substrates to form cyanohydrins. Recently, Lehninger (1950) reviewed the role of metal ions in enzyme systems. Since several heavy metals may react with cyanide, various enzymatic processes in the total respiratory metabolism may be altered. These alternatives should be kept in mind in considering the significance of the present results.

Cyanide insensitive respiration

Upon apparently complete inhibition of the cyanide sensitive respiration, there still remained a residual oxygen consumption. It is evident from Figures 1 and 2 that the cyanide insensitive respiration is relatively constant throughout metamorphosis, although different for each strain. In the normal strain the average cyanide insensitive oxygen consumption was 2.0 cu. mm./hr./pupa, whereas for the resistant strain it was twice as great. In Figure 5, by plotting original oxygen consumption against cyanide inhibited respiration, a line is obtained whose intercept on the ordinate represents the true value of the cyanide stable respiration.

The experimental values (Figs. 1 and 2) agree with the value obtained by such treatment of the data.

Although there are several enzymes, including cytochrome *b*, which are capable of reacting with oxygen and are not inhibited by cyanide, only for one of these, the "yellow enzyme" of Warburg and Christian (1932), are there sufficient data to estimate its significance in relation to the total respiration. The substrates undergoing oxidation through this enzyme are the hexosephosphates, citrate, glucose, and, to some extent, malate. Gourevitch (1937) reported a definite relationship in mammalian tissues between the quantity of flavin and the amount of cyanide insensitive respiration. Groen and Schuyl (1938) fed rats a flavin-free diet and found that the loss of flavin from the liver was accompanied by a corresponding reduction in the cyanide insensitive respiration. Normal and treated kidney, however, contained the same amount of flavin and the cyanide stable oxygen consumption was the same. Further evidence is found in the experiments of Pett (1936), who showed that when yeast is cultured in a medium containing cyanide, its flavin content is doubled and that correspondingly the cyanide insensitive respiration is also doubled. Thus, it is indicated that the absolute value of the cyanide stable respiration is directly related to the flavoprotein content of the tissue.

Although, as yet, flavin determinations have not been made on house fly pupae, the fact that the DDT-resistant strain has a greater cyanide insensitive respiration than the normal strain suggests the desirability of such a comparative study.

Comparison of the two strains.

The normal oxygen consumption of both strains of pupae during metamorphosis was approximately the same (Figs. 1 and 2).

It has been shown in Figure 3 that in all stages of development, except immediately before emergence of the adults, the pupae of the DDT-resistant strain have less cytochrome oxidase than the normal strain. At zero age the normal strain had approximately 3.5 times as much oxidase. Pupae one, two and three days old had 2.2 times, 2.4 times and 1.4 times as much oxidase, respectively. This suggests that in the resistant pupae a larger portion of the normal oxygen consumption passes through other respiratory systems, as is in fact demonstrated by the data given in Figures 1, 2 and 5, which reveal that the DDT-resistant pupae have twice the cyanide insensitive respiration of the normal strain.

As shown in Table I, oxygen consumption was inhibited by a given cyanide concentration to a greater extent in the normal strain than in the resistant strain. These results may be related to the finding that, as shown in Table II, the DDT-resistant pupae exhibit resistance to this poison.

The data in Table II also reveal that the lethal effect of a given cyanide concentration is dependent on the stage of development. In the normal strain, pupae one and two days old are least susceptible. This is in agreement with the fact that a larger portion of the normal oxygen consumption of these pupae is through the cyanide insensitive system. Of particular interest is the observation that, at a cyanide concentration of 3×10^{-4} *M*, 3 and 4 day old normal strain pupae mostly succumbed, whereas DDT-resistant pupae of the same age mostly survived. At this cyanide concentration the extent of inhibition in both strains was of the same

small magnitude. The possible mechanisms of this difference in survival of the two strains at these ages are speculative. They may be due to: (1) the dependence upon the cytochrome oxidase system for completion of certain developmental processes (Wolsky, 1937) which are stopped by the partial inhibition of the enzyme in the normal strain, whereas in the resistant strain, although the oxidase is also partially inhibited, these processes can proceed by the utilization of the flavin system; (2) more readily reversible inhibition of cytochrome oxidase in the resistant strain; (3) detoxification of cyanide by the resistant strain after dissociation of the inhibitor-enzyme complex. At this moment, we have no means of deciding which of these possible mechanisms, or combination of them, is actually employed.

It should also be noted that the mechanism of cyanide resistance in pupae may be different from that of DDT-resistance in adults. It was shown by Sacktor (1951) that DDT-resistant adults had approximately 50 per cent more oxidase than the normal strain. This suggests that one of the explanations for the resistance of adult house flies to DDT may be the greater oxidase activity, which will permit the continuance of essential physiological functions despite partial inhibition. In contrast, pupae may utilize a cyanide insensitive system for continuance of these functions. Further, the results of Sternburg and Kearns (1950), Sternburg, Kearns and Bruce (1950) and Perry and Hoskins (1950) in regard to the degradation of DDT by resistant strains must be considered. At present the mechanism of detoxification, or the possible role of DDE in preventing lethal effects of DDT is not known, and it is even possible that cytochrome oxidase is concerned in these mechanisms.

The present study suggests that DDT-resistance may be correlated with ability to maintain respiration in the face of partial inhibition of cytochrome oxidase. At the same time, the results draw attention to the complexity of the mechanisms concerned. These will require further investigation from the several other points of view noted above before a satisfactory interpretation can be reached.

Summary

1. Oxygen consumption follows a U-shaped curve during metamorphosis of normal and DDT-resistant strains of house flies. It is of the same order of magnitude in both strains.

2. Cytochrome oxidase activity during metamorphosis also follows a U-shaped curve. There is evidence that changes in oxygen consumption during this process are, in some respects, related to the activity of the oxidase.

3. A cyanide insensitive system, possibly flavin, and another cyanide sensitive system, probably tyrosinase, contribute to the total oxygen consumption. The latter system apparently contributes mainly during the early stages of metamorphosis.

4. The cyanide insensitive respiration of both strains remains relatively constant throughout development. The resistant strain has twice the cyanide insensitive respiration of the normal strain.

5. The DDT-resistant pupae have less cytochrome oxidase activity than normal pupae at all stages except immediately prior to emergence of the adult.

6. The pupae of DDT-resistant flies exhibit resistance to cyanide.

7. The effects of cyanide vary, depending on the developmental stage of the

pupae. A given concentration of the inhibitor produces different degrees of inhibition and mortality in pupae of different age. One of the possible explanations may be a change in the substrate being metabolized. Other possibilities are mentioned.

8. The possible mechanisms of resistance of pupae to cyanide are discussed. These may be: (a) a by-pass of the cytochrome system; (b) a difference in reversibility of enzyme-inhibitor complex or; (c) a detoxification of inhibitor. It is suggested that DDT-resistance may depend, in part, on similar factors.

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